Chapter 22

Molecular Osteology

Traditional methods for analyzing skeletal remains are based on examining the sizes and shapes of bones. Accelerating advances in other fields have given rise to a new set of methods that allow the osteologist to analyze the molecular constituents of bone. Under certain conditions, skeletal remains retain sufficient quantities of DNA, amino acids, proteins, and/or various isotopes to permit their recovery and analysis. The techniques used are often complex and require specially equipped laboratory facilities. Contamination and diagenesis can confound the results (Nicholson et al., 2002). However, the accuracy and precision of the results obtained may be superior to those of traditional methods, depending on the question. Molecular methods are thus becoming the methods of choice for various types of osteological analysis. Even so, these techniques are best applied as part of an overall osteological analysis rather than in isolation.

DNA analysis, in particular, allows the bioarchaeologist or forensic osteologist to address questions that are beyond the range of morphological methods. Such methods are also finding application in paleontology, as seen in the recovery of both mitochondrial and nuclear DNA from Neanderthals (see Section 22.2.4).

As molecular biologists develop ways to extract more information from modern DNA, the types of questions that can be addressed with ancient DNA are expanding as well. For reviews, see Kaestle and Horsburgh (2002) and Pääbo et al. (2004). Currently, there are four major questions about a deceased individual that DNA analysis of skeletal remains can potentially address: sex, pathology, ancestry, and individual identity. Isotopic techniques can shed light on diet and potentially residence patterns.

22.1 Sampling

The use of new high-tech molecular methods does not mean that the fundamentals of proper recovery, preparation, and documentation of skeletal remains can be ignored. Indeed, mistakes made during the sampling of material for molecular analysis can seriously compromise results. In addition to the standard set of recommendations for recovery and documentation of skeletal remains (Chapter 15), investigators wishing to obtain good samples for molecular analysis should consider the following:

- **Potential contamination.** If molecular analysis is to be employed on newly recovered skeletal material, recovery procedures should be modified to minimize contamination of

the remains by modern compounds and modern humans. The specific procedures will depend on the nature of the site, the available time and resources, and the type of analysis planned. For DNA analysis, these would ideally include using disposable latex gloves and hair nets, and sterilizing excavation tools. If this is not practical for the entire sample, a reasonable compromise may be to employ such procedures on the specific elements destined for DNA analysis. Remember that all specimens should be protected against contamination and degradation. The Armed Forces DNA Identification Laboratory (AFDIL) has particular guidelines that cover these topics and many others.

- **Taphonomic alteration.** The exposure of skeletal remains substantially alters their environment. This may, in turn, lead to further decay of their molecular constituents. Currently, little is known about the effects of preservatives and changes in temperature, humidity, moisture, and air circulation on the preservation of various biomolecules. Until more information is available, the wisest course is to minimize the magnitude of such changes as much as is practical. Consulting with the specialist who will be conducting the molecular analysis is advisable.
- **Provenience of sample.** The evaluation of the accuracy of molecular analyses depends in part upon proper documentation of the provenience of the samples. If molecular analysis is to be employed on newly recovered skeletal material, particular attention should be paid to properly documenting both the context of the remains and the excavation methods employed. Depending on the type of analysis planned, it may be advisable to obtain soil samples from the area surrounding the elements to be analyzed. Here again, consultation with the appropriate specialist is crucial.
- **Selection of sample.** Whether the molecular methods are to be applied to newly recovered skeletal remains or to museum collections, the choice of which specimens to sample must be made carefully. Because molecular techniques are typically destructive, specimens for sampling should be chosen to minimize the morphological information lost while maximizing the potential information gained in the molecular analysis (DeGusta and White, 1996). In order to adequately weigh these often-competing goals, it is imperative that the skeletal remains first be examined for signs of pathology, bone modification, and other morphological variations. Only then can an accurate assessment be made of the "morphological value" of the skeletal specimens and of the various portions of individual specimens. It is often useful for molecular analysis to be first attempted on nonhuman remains or on human specimens of dubious provenience to establish the feasibility of the method prior to the destructive sampling of more valuable specimens.
- **Pre-sampling documentation.** Removal of skeletal tissue for molecular analysis destroys information about the morphology of the bone, but this loss can be reduced greatly by proper documentation prior to sampling. The exact methods employed to record the morphology will depend on the anticipated degree of destruction and the importance of the specimen. Minimally, high-quality photographs (with a scale bar) should be taken prior to sampling. Molding and casting of specimens provide a three-dimensional record of the morphology, as does 3-D laser scanning. This, in combination with photographs and potentially also radiographs, minimizes the loss of information. Documentation only preserves morphological information if it remains permanently accessible, so casts and photographs of the relevant specimens (along with copies of the results of the molecular analysis) should be deposited properly and promptly in the appropriate archives.

22.2 DNA

Deoxyribonucleic acid (**DNA**) is the molecule of heredity. The genetic code in DNA is based on four chemical building blocks called nucleotides: adenine, cytosine, thymine, and guanine. These nucleotides can be thought of as forming a four-letter alphabet that spells out the assembly instructions for all the proteins that make up an organism. Almost every cell in a person's body has a complete copy of their DNA, and all these copies are essentially identical.

There is a tremendous amount of research aimed at extracting various types of information from DNA, in part because many diseases are thought to have a genetic component. In a biomedical context, specific diseases such as Huntington's disease and sickle cell anemia can be diagnosed based on DNA alone. The sex and general ancestry of an unknown individual can be determined from DNA. Comparisons of DNA samples can be used to establish identity and paternity. Brown (2000) provides an overview of the application of "ancient DNA" studies to human osteology.

The ability to determine sex, ancestry, disease status, and identity from DNA has obvious applications in osteology, forensics, archaeology, and even paleontology. These applications, though, all depend on the ability to obtain DNA from organic remains of varying antiquity. After an organism dies, the highly organized molecules of DNA degrade rapidly. The key conceptual breakthrough that led to the field of ancient DNA was the recognition that despite this decay, fragments of DNA are sometimes present in remains of great antiquity. With only a few modifications, existing techniques for isolating modern DNA can also be used to retrieve this DNA (Kolman and Tuross, 2000). These techniques originally required a large amount of preserved DNA, a criterion met only in cases of exceptional preservation (*e.g.,* mummies or ice-embedded animals).

22.2.1 PCR and methodology

Development of the **polymerase chain reaction** (PCR) made it possible to retrieve exponentially smaller amounts of DNA (Bartlett and Stirling, 2003). In order to analyze DNA, it is necessary to have a sufficient quantity of it. Using conventional techniques, even a dozen molecules of DNA are effectively invisible — they are too small to detect. The PCR acts as a "molecular photocopy machine" by making literally millions of copies of a section of DNA. This is referred to as amplification, and PCR can amplify a section of DNA starting from only a few original molecules. The large amount of DNA yielded by the method can then be analyzed quite easily using a variety of standard techniques. Using PCR, researchers were able to retrieve DNA from ancient skeletal remains (Hagelberg et al., 1989). This advance made ancient DNA methodology applicable to a broad range of questions in osteology.

The general methodology for obtaining and analyzing DNA from a bone or tooth involves three general stages. First, the DNA must be extracted and isolated. This involves reducing about a gram of bone or tooth to powder. The powder is then treated chemically to remove proteins and other compounds and to concentrate the DNA. Second, a predetermined section of the DNA is amplified using PCR. Finally, the resulting DNA sample is analyzed, typically by determining its nucleotide sequence. Knapp and Hofreiter (2010) examine the applicability of the "next generation sequencing" techniques to ancient DNA. Navascués et al. (2010) examine the problems inherent in comparing ancient DNA to modern DNA using analytic software designed according to panmictic assumptions (*i.e.,* contemporaneous populations of equally potentially interbreeding individuals).

22.2.2 Contamination

Despite the exciting potential of ancient DNA, the applications are still limited by methodological difficulties (Cooper and Poinar, 2000; Gilbert et al., 2005). The major problem is contamination by exogenous DNA (DNA not from the targeted individual). Living organisms are con-

stantly shedding DNA-bearing tissues in the form of skin cells, hair, saliva, and other secretions. Archaeological skeletal remains risk being contaminated by the DNA from organisms in the soil, microorganisms growing within the bones, excavators, curators, or even the DNA analysts themselves. The problem of contamination is worsened by the nature of PCR. The polymerase chain reaction preferentially amplifies well-preserved DNA molecules, which are more likely to be modern contaminants than truly ancient DNA. Because PCR produces large amounts of highly concentrated DNA, laboratories often encounter problems with the products of previous PCR reactions contaminating current work. Contamination is of extreme concern when attempting to retrieve DNA from ancient human remains, as humans are also the main source of exogenous DNA, making contamination more difficult to detect. Several published DNA sequences from very ancient remains are now widely held to be inauthentic (Lindahl, 1997). Because the materials used for the extraction of DNA are often unique, and the analysis is time consuming, independent replication of results is not always carried out. A number of techniques have been developed to reduce the chances of contamination occurring, and to increase the likelihood of the contamination being recognized. The independent verification of ancient DNA results is also becoming more common. So, whereas contamination will likely continue to be of concern, the body of reliable ancient DNA work will also continue to expand.

22.2.3 Taphonomy of DNA

Beyond a certain time period, perhaps 100,000 years (Poinar et al., 1996), no DNA is likely to be preserved in skeletal remains. Within that time range, the factors leading to preservation are not well understood. For example, some skeletal remains may yield DNA, whereas other remains, of similar antiquity or even younger, may not. The specifics of death, burial, and **diagenesis** (a change in the chemical, physical, or biological composition of bone subsequent to death; see Chapter 20) that result in the preservation of DNA in some cases, but not in others, are unknown. The preservation of DNA does seem to be primarily influenced by environmental conditions rather than by time, at least for remains younger than about 10,000 years (Parsons and Weedn, 1997). Bones and teeth that are macroscopically and microscopically well-preserved seem somewhat more likely to yield DNA. Empirical evidence also suggests that remains from colder regions may preserve DNA better than remains from warmer areas (Poinar et al., 1996). Eklund and Thomas (2010) examine the extent to which chemicals commonly used in conservation treatments of human and animal remains can damage DNA. Alaeddini et al. (2010) examine the effects/role of decomposition in DNA degradation and the potential impact it may have on forensic DNA profiling and identification.

Mitochondrial DNA (mtDNA)—the small portion of the genome that is inherited only from the mother—is easier to retrieve than nuclear DNA (Parsons and Weedn, 1997). This is likely due to the greater number of copies of mtDNA and perhaps its smaller, circular structure. Ho and Gilbert (2010) provide a review of the first decade of research into ancient mitochondrial genomics.

22.2.4 Applications

As molecular biologists develop ways to extract more information from modern DNA, the types of questions that can be addressed with ancient DNA will expand as well. Currently, there are five major questions about a deceased individual that DNA analysis of skeletal remains can potentially address:

- **What sex was this individual?** Methods of sexing skeletal remains based on morphology depend on the preservation of sexually dimorphic elements and have a significant error rate even for adult remains (Chapter 18). If DNA can be obtained, the sex of any

individual (regardless of age) can be determined with extremely high accuracy from even very fragmentary skeletal remains (Stone et al., 1996).

- **What diseases did this individual have?** A number of diseases are genetic in nature and could potentially be screened for in past populations using DNA analysis. Disease processes characterized by long-term infection by substantial densities of viral or bacterial pathogens might also be detected through recovery of the pathogen's DNA. As yet, very few studies of paleopathology have utilized DNA methods. The most notable application to date was the amplification of the *Mycobacterium tuberculosis* DNA from a Peruvian mummy to verify a pre-Columbian occurrence of tuberculosis in the New World (Salo et al., 1994).
- **With which ancestral population(s) did this individual have affinity?** A number of morphological techniques have been developed to assess the geographic affinity of skeletal remains, but they are of limited accuracy and require relatively complete remains (Chapter 21). Reliable methods to assess the populational affinities of skeletal remains are sorely needed in archaeology (to assess the relationships between past populations) and forensics (to provide information on an isolated skeleton that may lead to identification). DNA typing of skeletal remains has the potential to provide the best available information regarding the populational affinity of the individual. Mitochondrial DNA (mtDNA) is a small portion of the human genome that is inherited only from the mother. Several regions of mtDNA are highly variable within modern humans, and the sequencing of these regions can permit the estimation of the ancestral maternal population (Connor and Stoneking, 1994). Archaeologically, these techniques have been used to assess the relationships of prehistoric New World populations with various modern Native American groups (Stone and Stoneking, 1993; Hauswirth et al., 1994; Kaestle, 1995). Wallace and Torroni (2009) examine the interrelationships and ancestry of the three major linguistic groups of Native Americans. Forensically, analysis of mtDNA has been used to help identify the remains of U.S. military personnel in Vietnam (Holland et al., 1993).
- **Who was this individual?** A relatively common problem in forensic osteology is to establish the identity of a skeletonized individual. In some cases, a possible identity will be established based on other clues (*e.g.,* a recently missing person of similar age and sex), but confirmation is needed. DNA analysis is the best method for testing hypotheses about the identity of skeletal remains. The general approach is to compare the DNA from the skeleton with the DNA of the presumed relatives. For a number of variable regions of the DNA, the odds of a match between unrelated individuals are extremely low. Exactly how low is a matter of debate for cases involving blood samples from living individuals (Devlin et al., 1994), but in osteological contexts this is rarely if ever a concern. Steadman (2003) provides a review of mitochondrial DNA analysis and histomorphology. As she notes for the forensic context, DNA can build a genetic profile and result in individuation, but it cannot construct a biological profile of the individual. DNA typing has been used to identify skeletonized individuals in contexts involving mass deaths (the Branch Davidian incident in Waco, Texas; Houck et al., 1996), mass graves (Guatemala and former Yugoslavia; Boles et al., 1995; Primorac et al., 1996), remains of military personnel (Vietnam; Holland et al., 1993), war criminals (Josef Mengele; Jeffreys et al., 1992), historic figures (the Romanov family; Ivanov et al., 1996), and numerous forensic cases involving murder victims (*e.g.,* Hagelberg et al., 1991; Sweet and Sweet, 1995).
- **To which individuals was this individual related?** Even though the determination of familial relationships is most applicable in forensic or historical contexts, archaeological analysis of mortuary rituals and burial practices can often be advanced if the general relationships of the interred individuals can be established (Stone and Stoneking, 1993). Establishing the familial relationships between individuals in the same prehistoric population requires more detailed analysis than is usually attempted.
- **To what species did this individual belong?** Identification at the species level, some-

times a problem with extremely fragmentary remains, is also made possible through DNA analysis. However, the determination of whether fragmentary skeletal remains are human or nonhuman can usually be done quickly, cheaply, and accurately by visual inspection of the morphology, rendering DNA analysis unnecessary for this question in most osteological contexts. In paleontological settings, though, morphological methods for determining the species affiliation of hominid fossils often produce ambiguous results. In the case of Neanderthals, for example, paleoanthropologists working on skeletal remains have disagreed about whether there was gene flow between Neanderthals and contemporaneous early modern humans. A comparison of Neanderthal DNA with the DNA of early modern humans is providing a crucial test of hypotheses about their relations. Both mtDNA and nuclear DNA have been retrieved from a number of Neanderthal and early modern human remains, and substantial progress has been made toward assembling the complete Neanderthal genome. In May, 2010, Green et al. published their work sequencing 4 billion nucleotides from three Neanderthal individuals to map about 60% of the entire Neanderthal nuclear genome. The results from both nuclear DNA and mtDNA generally support the separation of these two lineages, but the specifics remain a matter of debate due to differing criteria for recognizing gene flow. For instance, Green and colleagues found support for low levels of gene flow (about 1–4%) from Neanderthals to modern humans at some point after the divergence of African and non-African lineages, but before the divergence of European and Asian lineages. Even though the processes of fossilization and DNA degradation preclude the application of DNA techniques to most of the fossil record, questions about species and evolutionary relationships within the last few hundred thousand years are now beginning to be addressed using this method.

22.3 Amino Acids

Amino acids are the chemical building blocks of proteins in all living organisms. Each type of amino acid comes in two mirror image forms: the D form and the L form. All amino acids incorporated into a protein are in the L form, but over time they gradually convert into the D form, a process known as racemization. Attempts to use the ratio of D to L forms as an absolute dating method have generally failed, as this ratio is significantly affected by diagenesis, but the technique has found two applications in modern osteology.

First, it has been suggested that the D:L ratio of aspartic acid in teeth is indicative of age-at-death (Ohtani and Yamamoto, 1991, 1992; Ohtani, 1995; Carolan et al., 1997). However, the error of the estimate is figured at about ±15 years, and since the ratio is affected by diagenesis, the method is only applicable in modern contexts. Due to these limitations, the application of amino acid racemization for establishing age-at-death is of little use.

Second, Poinar et al. (1996) have proposed that the degree of racemization of aspartic acid in skeletal remains is correlated with the preservation of DNA. Beyond a certain degree of racemization, DNA was unlikely to be amplified. This technique takes advantage of the sensitivity of the racemization process to environmental factors, since it is just those factors which speed the decay of DNA. The main use of amino acid racemization in ancient DNA work is to assess the prospects of retrieving DNA and to help confirm the authenticity of the DNA obtained, although newer techniques are rapidly emerging (Green et al., 2009).

22.4 Isotopes

The documentation of diet in the past provides the context for studies of growth, stress, disease, and subsistence activities (Larsen, 1997). Questions regarding diet have traditionally been approached via morphological assessment of dental and skeletal remains, as described in Chapter 21. Increasingly, however, isotope studies are becoming the method of choice for investigating past diets, with the potential to also shed light on group affinities. In general, these methods exploit the variation in isotopic ratios preserved in bone and teeth to infer the composition of past diets and geological surroundings. See Section 21.3.3 for a discussion of isotopic analyses in osteology.

Suggested Further Readings

Cooper, A., and Poinar, H. N. (2000) Ancient DNA: Do it right or not at ALL. *Science* 289: 1139.

A sobering call to dismiss premature optimism about ancient DNA and to objectively gauge all ancient DNA results using 9 suggested "criteria of authenticity."

Gilbert, M. T. P., Bandelt, H.-J., Hofreiter, M., and Barnes, I. (2005) Assessing ancient DNA studies. *Trends in Ecology & Evolution* 20:541–544

An argument against adopting the 9 criteria of authenticity for ancient DNA, advocating instead "a critical consideration of all available information."

Graur, D., and Li, W.-H. (2000) *Fundamentals of molecular evolution* (2nd ed.). Sunderland, MA: Sinauer. 481 pp.

A textbook that presents both the essential information on DNA as well as the use of DNA in studies of evolution.

Green, R. E., and 55 others (2010) A draft sequence of the Neandertal genome. *Science* 328: 710–722.

The announcement of the mapping of the Neanderthal nuclear genome, with a good discussion of gene flow between modern humans, Neanderthals, and earlier hominids.

Herrmann, B., and Hummel, S. (Eds.) (1994) *Ancient DNA*. New York, NY: Springer-Verlag. 263 pp.

This edited volume provides an overview of the field, with an emphasis on techniques rather than applications.

Ho, S. Y. W., and Gilbert, M. T. P. (2010) Ancient mitogenomics. *Mitochondrion* 10:1–11.

A review of the first decade of ancient mitochondrial genomic research.

Kelman, L. M., and Kelman, Z. (1999) The use of ancient DNA in paleontological studies. *Journal of Vertebrate Paleontology* 19:8–20.

An excellent introduction to the subject with good cautions regarding contamination.

Knapp, M., and Hofreiter, M. (2010) Next generation sequencing of ancient DNA: Requirements, strategies and perspectives. *Genes* 1:227–243.

An examination of some of the most recent techniques in DNA sequencing and the degree to which each may be suitable to sequencing ancient DNA.

Krings, M., Stone, A., Schmitz, R. W., Krainitzki, H., Stoneking, M., and Pääbo, S. (1997) Neandertal DNA sequences and the origin of modern humans. *Cell* 90:19–30.

The first successful application of DNA methods to the human fossil record.

Navascués, M., Depaulis, F., and Emerson, B. C. (2010) Combining contemporary and ancient DNA in population genetic and phylogeographical studies. *Molecular Ecology Resources* 10:760–772.

An enlightening discussion of the problems inherent in comparing DNA from different time periods using algorithms designed for comparing contemporaneous individuals from a panmictic population.

Pääbo, S., Poinar, H., Serre, D., Jaenicke-Després, V., Hebler, J., Rohland, N., Kuch, M., Krause, J., Vigilant, L., and Hofreiter, M. (2004) Genetic analyses from ancient DNA. *Annual Review of Genetics* 38: 645–679.

A critical analysis of the state of ancient DNA research, with a consideration of what is currently possible and what can be reasonably expected from future research.

Parsons, T. J., and Weedn, V. W. (1997) Preservation and recovery of DNA in postmortem specimens and trace samples. In: W. D. Haglund and M. H. Sorg (Eds.) *Forensic taphonomy: The postmortem fate of human remains.* Pp. 109–138. Boca Raton, FL: CRC Press.

A review of the preservation of DNA in postmortem samples, including both forensic and ancient skeletal remains, and how it can be retrieved and analyzed.

Stone, A. C., and Stoneking, M. (1993) Ancient DNA from a pre-Columbian Amerindian population. *American Journal of Physical Anthropology* 92:463–471.

One of the few actual analyses of a prehistoric skeletal sample using DNA methods.

Stoneking, M. (1995) Ancient DNA: How do you know when you have it and what can you do with it? *American Journal of Human Genetics* 57:1259–1262.

Addresses the standards needed in ancient DNA work and identifies productive areas of future research.

Wallace, D. C., and Torroni, A. (2009) American Indian prehistory as written in the mitochondrial DNA: A review. *Human Biology* 81:509–521.

An examination of the mitochondrial evidence for addressing the question of the number of migration events for aboriginal American Indians.